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G. Jansen, B. Deddens, M. Wilkinson, D. van der Waaij

The Influence of *Enterococcus faecalis* on the Morphology and the Antibody-Binding Capacity of the Intestinal Bacteria of Ten Healthy Human Volunteers

Summary: The influence of *Enterococcus faecalis* on the morphology of the bacterial cells which make up the gut microflora and on the levels of circulating IgG bound to the gut microflora was assessed. After 29 days of pretreatment monitoring, ten healthy human volunteers ingested 10^7 viable cells of *E. faecalis* three times daily, for 21 days. After this treatment another 21 days of follow-up completed the study. Each volunteer delivered eleven faecal samples during the entire study period of 71 days with a 7 day interval. Before and after the faeces sampling period, blood samples were collected from all volunteers. The influence of the ingestion of *E. faecalis* on the morphology of the gut microflora was measured by image analysis. In addition, the binding of circulating IgG to intestinal bacteria in all intermediate faecal samples was measured by means of quantitative immunofluorescence. The oral administration of *E. faecalis* resulted in a significant change of the morphological composition of the gut microflora and in a significant decrease in IgG-binding capacity of the gut microflora.

Introduction

In 1989, Fuller defined probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" [1]. In this definition, no statement concerning the mechanisms by which the intestinal bacteria may exert their influence on the health status of the host organism is included. Currently, a substantial bibliography dealing with the concept of probiosis is available, especially fundamental ecological knowledge concerning the intestinal microflora [2-4]. Besides ecological effects on the gut microflora, probiotics may exert a modulation of the immune system of the host. After bacterial translocation [5] from the gut microflora to as far as the mesenteric lymph nodes and other lymphoid organs, a systemic immune response [6] may be induced. The use of *Enterococcus* spp. as a probiotic immunomodulator in humans seems attractive since Rusch et al. observed a significant decrease in the risk of infection associated with the oral intake of viable enterococci [7]. In this study, two questions will be addressed. Firstly, in a group of ten healthy human volunteers, does the oral intake of 10^7 viable cells of *E. faecalis* three times daily, for 21 days, result in a change in the morphological composition of the gut microflora. Secondly, if upon such treatment a change in the morphological composition of the gut microflora is observed, is this effect accompanied by an alteration in IgG-binding capacity of the indigenous gut flora?

The relevance of the first question follows from observations of Meijer et al. who found a significant decrease in morphological diversity of the gut microflora after ceftriaxone therapy [8]. Because antimicrobial chemotherapy is associated with a decreased colonization resistance [9,10], the morphological diversity of the bacteria present in the

gut microflora may be regarded as indicative for the stability of the intestinal ecosystem. Therefore, although the morphological diversity bears no relationship to the phylogenetic composition of the gut microflora it can be used as a descriptive parameter of the stability of the gut microflora. Analysis of the morphological composition of the gut microflora was performed using the image analysis system and algorithms previously described [11] by Meijer et al. It should be emphasized that this method of analysis includes both the culturable and non-culturable fraction of the gut microflora. Therefore, the information obtained with this method probably does not apply directly to the culturable fraction of gut microflora.

The relevance of the second question is apparent since, in a previous pilot study, we observed a significant decrease in titres of circulating IgG directed against *E. faecalis* after oral administration of this microorganism in a group of ten healthy human volunteers [12]. The difference between the levels of IgG against the gut microflora in a serum sample obtained prior to the treatment and a serum sample obtained after the treatment may be indicative of changes in both the antigenic composition of the gut microflora and the IgG repertoire present in the circulation. Levels of circulating IgG bound to the bacteria of the gut microflora were measured by means of quantitative immunofluorescence using a FITC-labelled goat anti-human F(ab')₂ conjugate [13].

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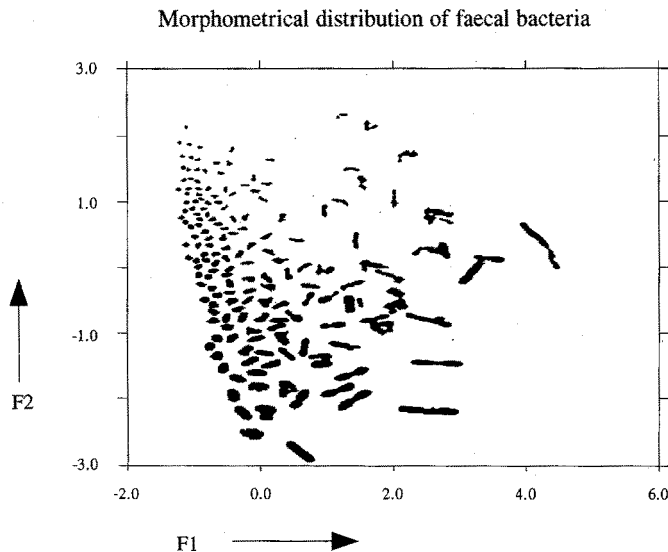


Figure 1: Morphometrical plot of bacterial objects present in normal human faeces. Objects in the morphometrical region are represented as bacteria instead of symbols. For reasons of clarity some bacteria are omitted. Each object is positioned according to its (F1,F2)-score. F1 and F2 are (independent) principal components.

Materials and Methods

Volunteers: Ten healthy human volunteers, eight male and two female, aged 22–61 years, entered this study after they had given written informed consent. This experiment was reviewed and approved by the Medical Ethics Committee of the University Hospital Groningen. None of the volunteers had received antimicrobial chemotherapy during the 2 months preceding the study, and no one had altered dietary habits in the same period. During the same period no volunteer showed any symptoms of gastrointestinal disorders. The only type of medication allowed during this study were contraceptive hormone preparations.

Sampling and experimental design: The study had a sequential design. During a relatively long (i.e. 29 days) observation period, baseline values of the parameters under consideration were obtained for each volunteer. Consequently, each volunteer acted as his/her own negative control. The total study period lasted 71 days. Per volunteer eleven faecal samples were collected with a 7 day interval. Sampling was started on day 1. Consequently, a total of 110 faecal samples were obtained. All faecal samples were stored at -20°C until processing. Before the sampling started, each volunteer delivered a sample of blood. After a pretreatment monitoring period of 29 days, *E. faecalis* was administered to all volunteers. The dosage regimen comprised an intake of 10^7 viable cells of *E. faecalis* (Symbioflor I[®], SymbioPharm, Herborn-Dill, Germany) three times daily, for 21 days. Viability was confirmed by triplicate inoculation of 1 ml of a 10^4 to 10^{10} -times diluted Symbioflor I[®]-suspension on sheep blood agar. Colonies were counted after 24 h of incubation at 37°C .

After treatment another 21 days of follow-up completed the study period. Finally, at day 71, a second blood sample was obtained from each volunteer. In each faecal sample, the morphology of all faecal objects was measured in duplicate. Furthermore, for each volunteer, the binding of IgG present in the blood samples to a person's own faecal samples was assayed in duplicate.

The rationale for this design is that the first serum sample may contain – apart from a variety of other immunoglobulins – circulating IgG against the gut microflora of the untreated host. This serum, therefore, serves as a reference. Because the immunosuppressive capacity of *E. faecalis* has previously been established [12], the second serum sample, which was obtained after treatment, may contain a partly different antibody repertoire. Consequently, this serum serves as an indicator for changes in the antibody binding capacity of the gut microflora.

Serum: Blood samples were obtained by means of venous puncture and were maintained at room temperature for 1 h to ensure proper clotting. Thereafter, the blood samples were centrifuged in a centrifuge type TJ-6 (Beckmann, Palo Alto, California, USA) for 10 min at $1,490 \times g$. Finally, the serum was stored at -20°C in 0.2 ml aliquots.

Preparation and analysis of the immunofluorescence slides: Though the preparation of immunofluorescence slides has been described previously [13], some improvements have been made prior to this study. Half a gram of faeces suspended in 4.5 ml demineralized water containing 0.5% Tween-80 (Merck, Darmstadt, Germany) and homogenized on a vortex mixer for 2 min. Thereafter, the faecal suspension was centrifuged for 10 min at $1,490 \times g$ in a Beckmann TJ-6 and the supernatant was collected. Twelve-well slides (Immunocor, Limoges, France), were degreased and cleansed by sonication in a Branson 32 (Branson Ultrasonics B.V., Soest, The Netherlands) for 10 min using a 3% (v/v) Teepol[™] (Shell Chemistry N. V., The Netherlands) solution. The slides were then coated with a 10% (v/v) poly-L-lysine solution (Sigma Diagnostics, St. Louis, USA) to ensure proper adhesion of the faecal bacteria to the microscopic slide. Ten μl of the diluted faecal supernatant (1:25 in phosphate buffered saline (PBS) with pH 7.2 and a temperature of 37°C) was pipetted in two wells on the slide. After 10 min of drying at 37°C the slides were fixed for 10 min using a 50% (v/v) solution of ethanol in demineralized water. A fixative containing 50% (v/v) was chosen because fixatives with 70% (v/v) or 96% (v/v) ethanol increased the amount of debris in the microscopic field considerably. Although the debris is recorded under phase-contrast illumination, it did not emit any detectable light under ultra-violet illumination. Thereafter, one well was incubated for 45 min with 20 μl diluted (1:10 PBS with pH 7.2 and a temperature of 37°C) reference serum and the other well with the diluted indicator serum. Next, the slides were washed three times for 5 min with PBS of 37°C . Then each well was incubated with 20 μl of a solution (1:1000 in PBS) of fluorescein isothiocyanate (FITC)-conjugated goat anti-human F(ab')₂ IgG (Kallestad, Texas, USA) for 1 h at 21°C in a dark, moist chamber. Finally, the slides were washed three times in PBS of 37°C and mounted with a cover slip and two drops of mounting fluid (glycerol/TrisHCl 50% (v/v) pH 8.6). Slides were stored at 4°C until further processing. The slides were read by an image analysis system which consists of an Orthoplan epifluorescence-microscope (Leitz, Germany) equipped with a Fairchild CCD 5000/1 camera (Loral Fairchild Systems Inc., Sunnyvale, California, USA). The camera is connected to a Compaq Deskpro 80486 microcomputer with 8 Mb RAM and a MVP/AT image processing board (Matrox Ltd., Quebec, Canada). Furthermore the system is equipped with a camera-exposure and UV-illumination control board [14] by means of which the CCD camera exposure time under UV-illumination was set at 4.2 s. Using this exposure time, very low levels of emitted light could be quantified. Furthermore, the software used to control the data-acquisition process contains an option which enables

the operator (after visual confirmation) to discard objects from the analysis which are clearly not of bacterial origin.

Morphometrical analysis of the faecal preparations: Three morphometrical parameters per faecal object (F1, F2 and F3) were calculated from a total of 1,500 to 3,000 objects per analysis. These parameters were previously derived by Meijer et al. [15]. F1, F2 and F3 are principal components which explain 98.9% of the morphometrical variability and which are independent. In Figure 1 the F1,F2-scores of the bacterial objects present in a normal gut microflora are depicted. The diversity in the distribution of the faecal objects in the F1xF2xF3-space is quantified by means of the morphometrical entropy (S). When the distribution of faecal objects over the F1xF2xF3-space is homogeneous, S becomes large and when the majority of the faecal objects is confined to a small area of the F1xF2xF3-space, the value of S becomes small. For each faecal sample the value of S was assessed in duplicate.

Fluorimetical reading of the immunofluorescence slides: The levels of IgG directed against the gut microflora were quantified. Levels of IgG were expressed as the natural logarithm of the median fluorescence level of 1,500 to 3,000 FITC-labelled faecal objects minus the median fluorescence level of 1,500 to 3,000 unlabelled faecal objects. The logarithm was used to improve the normality of the distribution of the fluorescence levels. Each serum was assayed in duplicate and PBS instead of serum served as a negative control.

Results

In Figure 2 the mean morphometrical entropy (S) in the population is plotted as a function of time. The shaded zone indicates the 95% confidence interval calculated from the 50 S-values obtained during the first 29 days of the study period. Consequently, this interval accounts for the assay error, the interindividual differences and the longitudinal fluctuations of S. At day 50 and day 64 significantly lower values of S were observed. Visual examination of F1,F2-plots of the gut microflora at day 50 and 64 did not reveal a clear shift in morphology of the faecal objects in these samples from the "rod"-area (large F1, small F2) to the "coccoid"-area (small F1, large F2). During the convalescence period fluctuations in S were large. The error bars account for the assay error and the interindividual differences. From Figure 2 it can be seen that, during the pretreatment period, the variance (i.e. interindividual differences provided a constant assay error is assumed) is reasonably constant. This observation is indicative of a fairly constant distribution of S over the population. At day 43 and day 70, errors tend to be larger. The population's S at these days is therefore less reliable. The relatively small errors in the population's S at day 50 and at day 64 add to the reliability (and therefore the significance) of these aberrant S-values.

In Figure 3 the course of the mean levels of circulating IgG bound to gut flora in the ten sera obtained before sampling (solid line) and in the ten sera obtained at the end of the sampling period (dotted line) are depicted. Comparing both patterns of IgG bound to bacteria of the gut microflora by means of Student's t-test indicates that significant ($p < 0.05$) differences between the unchallenged sera

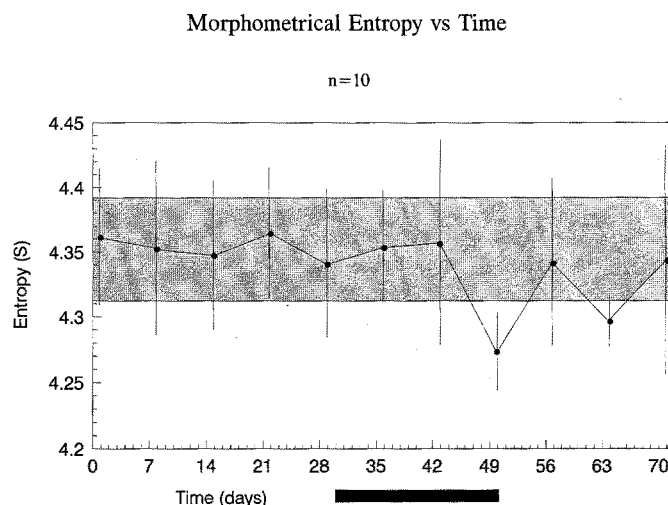


Figure 2: Mean morphometrical entropy (S) in a group of ten healthy human volunteers before, during and after treatment with 10^7 viable cells of *Enterococcus faecalis* three times daily for 21 days. The treatment period is indicated by a solid bar. The shaded zone indicates the 95% confidence interval calculated from all S-values obtained during 29 days of observation. Error bars represent the standard error of the mean.

(which were obtained before faeces sampling) and the challenged sera (which were obtained after faeces sampling) appear on day 36 and on day 43 of the trial. Furthermore, a significant ($p < 0.05$) difference is observed at day 64. During the pretreatment period, the mean levels of IgG bound to gut microflora in the challenged and the un-

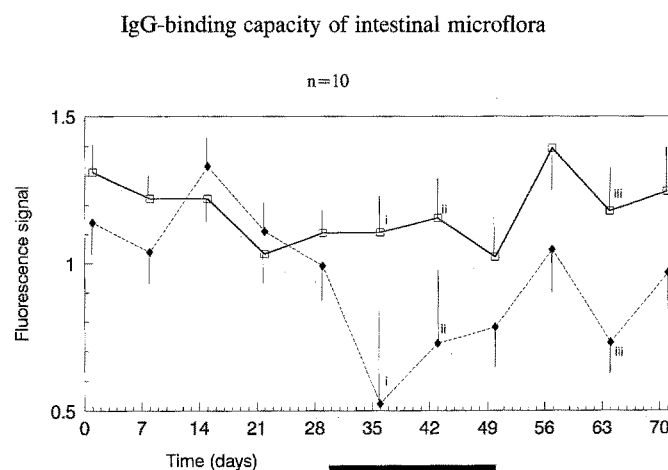


Figure 3: Mean levels of circulating IgG against intestinal microflora in sera obtained before faecal sampling (solid line) and in sera obtained after faecal sampling (dotted line) in a group of ten healthy human volunteers. Treatment comprises 10^7 viable cells of *Enterococcus faecalis* three times daily for 21 days. The treatment period is indicated by a solid bar. Equally marked pairs of mean IgG-levels were found to differ significantly ($p < 0.05$) when Student's t-test was employed. Error bars represent the standard error of the mean.

challenged sera are indistinguishable. Also, the errors in these IgG-levels are of comparable magnitude. This finding implies that during the observation period no differences in magnitude and distribution of levels of IgG bound to the gut microflora are detectable between both the challenged and the unchallenged series of sera. The large error in the levels of IgG directed against gut microflora in the challenged sera at day 36 and day 43 of the study period implies that substantial interindividual differences were observed. Although no significant difference between the levels of circulating IgG bound to the gut microflora between the challenged and the unchallenged sera could be detected at day 50, 57 and 71 the difference in levels of IgG between the challenged sera remains lower than in the unchallenged sera during the entire convalescence period. During the follow-up period, the errors in the levels of IgG directed against the gut microflora between both the challenged and the unchallenged sera were decreased to a size comparable to their original (i.e. pretreatment) value.

Discussion

When describing the morphology of the gut microflora, the morphometrical entropy (S) is a rather insensitive parameter. This becomes obvious when one realizes that half a gram of faeces contains over 10^{11} bacteria, while only 1,500 to 3,000 bacteria are evaluated in the analysis. Therefore it was remarkable that a small but significant effect in S was observed (Figure 2). The low sensitivity of S as a descriptive parameter of the morphology of the gut microflora may account for the observation that the first significant decrease of S was recorded after 21 days of treatment. In other words, although it cannot be concluded from these data, ecological disturbance may already have been in progress for some time before it was detected.

Secondly, the effect of the treatment comprises a decrease in S. This may seem contradictory since removal of some parts of the gut microflora – e.g. after antibiotic treatment [8] – results in a decrease of S, while in this study the gut microflora was supplemented with enterococci. Considering the relatively small number of enterococci administered, it seems unlikely that the addition of coccoid shaped bacteria accounts for the decrease in S. The observed effect is probably caused by secondary ecological disturbances following the oral feeding of *E. faecalis* into the intestinal ecosystem. However, it needs to be emphasized that on the basis of the morphometrical analysis of the gut microflora, no conclusions regarding bacterial species may be drawn.

In Figure 3, the solid line functions as a reference template, indicating the mean binding of circulating IgG (using sera obtained prior to treatment of the host with *E. fae-*

calis) with the gut microflora of ten healthy volunteers during 11 consecutive weeks. The dotted line indicates the IgG binding capacity of the same series of faecal samples using sera obtained after sampling.

In a previous study [12] a significant lowering of the level of circulating IgG against *E. faecalis* after oral treatment with this strain was observed. Therefore, it is obvious that the treatment results in a – partially – altered IgG-repertoire, at least with respect to the fraction of IgG directed against *E. faecalis*-related antigens. Because also an influence of the treatment on the morphological composition of the gut microflora *sec* is recorded (Figure 2), it is clear that the effect depicted in Figure 3 is due to at least two fundamentally different processes.

However, the differences between IgG-binding capacities obtained with challenged and unchallenged serum *within* each faecal sample appear to be significant at day 36, 43 and 64. These differences can only be due to differences in IgG repertoire between each pair of sera (both challenged and unchallenged) because the underlying antigenic substrate (the gut microflora) is identical for each pair of sera. The data obtained in this study demonstrate that the suppressive effect of the *E. faecalis* strain on the levels of circulating IgG does extend to other – gut microflora associated – antigens. This effect clearly results from the treatment and is probably not due to extensive changes in antigenic composition of the gut microflora because the mean IgG-binding capacity of the gut microflora, using the unchallenged sera, remains fairly constant over the entire period of study. The mechanism responsible for this decrease in binding IgG against bacteria of the gut microflora is not clear from these data.

In conclusion, oral treatment with 10^7 viable cells of *E. faecalis* three times daily for 21 days lowers the morphological diversity of the gut microflora. The bacterial ecosystem which inhabits the digestive tract is therefore probably more easily colonized by (potentially) pathogenic bacteria. This clearly is a non-probiotic effect. On the other hand, the treatment effectuates a lowering of the IgG-binding capacity of a myriad of antigens beside those related to the *Enterococcus* strain itself. This effect may be responsible for the anti-inflammatory effect which was observed earlier by Rush et al. [7]. We therefore conclude that the probiotic potential of this strain probably lies in its immune modulating capacity and not in its – presumed – stabilizing effect on the gut microflora itself.

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Zusammenfassung: Einfluß von *Enterococcus faecalis* auf die Morphologie und Antikörper-Bindungs-kapazität der Darmbakterien von zehn gesunden freiwilligen Probanden. Der Einfluß von *Enterococcus faecalis* auf die Morphologie der Bakterienzellen der Darmflora und auf die Bindungsfähigkeit von IgG an die Darmflora wurde bestimmt. Nach 29 Tagen Beobachtungs-Vorphase nahmen 10 freiwillige gesunde Versuchspersonen 21 Tage lang dreimal täglich 10^7 lebende Zellen von *E. faecalis* ein. Die Studie wurde nach einer weiteren Beobachtungszeit von 21 Tagen beendet. Während der gesamten Studienphase von 71 Tagen lieferte jeder der Probanden 11 Stuhlproben in siebentägigen Abständen ab. Vor und

nach der Gewinnung der Stuhlprobe wurden von allen Probanden Blutproben entnommen. Der Einfluß der Einnahme von *E. faecalis* auf die Morphologie der Mikroflora des Darmes wurde durch Bildanalyse beurteilt. Zusätzlich wurde die Bindungsfähigkeit von Serum-IgG an die Darmbakterien bei allen Stuhlproben der mittleren Studienphase mittels quantitativer Immunfluoreszenz bestimmt. Die orale Applikation von *E. faecalis* führte zu einer signifikanten Veränderung der Zusammensetzung der Darmflora gemessen an ihrem morphologischen Erscheinungsbild. Zugleich nahm die Fähigkeit der Darmflora zur Bindung von IgG signifikant ab.

References

- Fuller, R.: Probiotics in man and animals. J. Appl. Bacteriol. 66 (1989) 365–378.
- Freter, R., Brickner, H., Botney, M., Cleven, D., Aranki, A.: Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect. Immun. 39 (1983) 676–685.
- Freter, R., Stauffer, E., Cleven, D., Holdemann, L. V., Moore, W. E. C.: Continuous flow cultures as *in vitro* models of the ecology of large intestinal flora. Infect. Immun. 39 (1983) 666–675.
- Freter, R., Brickner, H., Temme, S.: An understanding of colonization resistance of the mammalian large intestine requires mathematical analysis. Microecol. Ther. 16 (1986) 147–155.
- Steffen, E. K., Berg, R. D., Deitch, E. A.: Comparison of the translocation of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes. J. Infect. Dis. 157 (1988) 1032–1037.
- Perdigon, G., Nader de Marcias, M. E., Alvares, S., Oliver, G., de Ruiz Holgado, A. P.: Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophilus*. Immunology 63 (1988) 17–23.
- Rusch, V., Kalinski, S., Schneider, H.: *Streptococcus faecalis* vaccine – results of double blind placebo studies. Microecol. Ther. 16 (1986) 59–74.
- Meijer, B. C., Kootstra, G. J., Geertsma, D. G., van der Waaij, D.: Effects of ceftriaxone on faecal flora: analysis by micromorphometry. Epidemiol. Infect. 106 (1992) 513–521.
- van der Waaij, D., Berghuis-de Vries, J. M., Lekkerkerk-van der Wees, J. E. C.: Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J. Hyg. Camb. 69 (1971) 405–411.
- van Saene, H. K. F., Stoutenbeek, C. P., Geitz, J. N., Hart, C. A.: Effect of amoxycillin on colonisation resistance in human volunteers. Microb. Ecol. Health Dis. 1 (1988) 169–177.
- Meijer, B. C., Kootstra, G. J., Wilkinson, M. H. F., van der Waaij, D.: A theoretical and practical investigation into the characterization of bacterial species by image analysis. Binary Comp. Microbiol. 2 (1990) 21–31.
- Jansen, G. J., Deddens, B., Wilkinson, M. H. F., van der Waaij, D.: Significant decrease of titres of circulating IgG after oral intake of a preparation of *Enterococcus faecalis* in a group of ten healthy volunteers. Infection 21 (1993) 153–154.
- Jansen, G. J., Wilkinson, M. H. F., Deddens, B., van der Waaij, D.: Statistical evaluation of an improved quantitative immunofluorescence method of measuring serum antibody levels directed against intestinal bacteria. J. Microbiol. Meth. 17 (1993) 137–144.
- Wilkinson, M. H. F., Jansen, G. J., van der Waaij, D.: Very low level fluorescence detection and imaging using a long exposure charge coupled device camera. In: Bach, P., Poole, P., Mottley, J. (eds.): Microscopic imaging, fluorescence and microinjection in biotechnology. Plenum Publishing Corporation, London 1993, pp. 221–230.
- Meijer, B. C., Kootstra, G. J., Wilkinson, M. H. F.: Morphometrical parameters of gut flora in human volunteers. Epidemiol. Infect. 107 (1991) (a) 383–392.